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PRINCIPAL INVESTIGATOR: Stephen Dewhurst, Ph.D.

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Rochester, NY

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14. ABSTRACT
There is a pressing need to develop new treatments for breast cancer. Oncolytic, conditionally-replicating adenoviruses (CRADs) represent one such approach. Our objective to develop new CRADs containing mutant DNA polymerases with a high functional dNTP requirement; we hypothesize that these vectors will replicate selectively in tumor cells. We have now shown that the thermostable Pfu DNA polymerase can be used as a surrogate for the adenovirus DNA polymerase, and we have used this approach to identify new amino acid residues that affect dNTP binding and utilization (residues Y690, M689 and G688 in adenovirus polymerase). We have also shown that mutations which drastically alter the dNTP binding efficiency of the adenovirus DNA polymerase give rise to replication-defective viruses, when substituted into the backbone of an intact adenovirus vector. In contrast, some mutations with more modest effects on dNTP binding efficiency (e.g., the I664V mutation) are compatible with virus replication and permit the recovery of infectious virus. In year two, we will develop and test additional CRADs with mutant DNA polymerases, and we will then examine their ability to selectively replicate in, and lyse, breast cancer cells.

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Adenovirus, Conditionally-replicating adenovirus vector, DNA polymerase, dNTP binding, oncolytic virus

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FOREWORD

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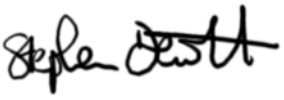
N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.



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PI - Signature **Date**

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INTRODUCTION

The development of effective treatments for breast cancer may require an array of different approaches, including gene therapy-based methods. Oncolytic, conditionally-replicating adenoviruses (CRADs) represent one such method, which can be used either alone or in combination with other treatments (including radio-, chemo- or immuno- therapies). While initial clinical studies of oncolytic CRADs have shown that these vectors are generally safe and well tolerated, additional improvements will be necessary to make oncolytic CRADs more effective. Thus, the key goal of this project is to develop more effective oncolytic CRADs, by using a highly flexible approach that can be readily integrated into, or combined with, other approaches in order to develop an optimal therapeutic product.

BODY

Approved Tasks

The following tasks were outlined in the final, modified approved statement of work for this grant:

Task 1. To identify mutated derivatives of the adenovirus DNA polymerase (Ad pol) that have a substantially reduced dNTP binding affinity, but normal catalytic activity under conditions of substrate excess. (Months 1-12).

A. Mutation of selected polymerase residues within Ad pol using site-directed mutagenesis. (Months 1-9). We will create and test a series of conservative substitutions at pol residues known to affect dNTP utilization. Mutations to be created and analyzed with include conservative changes at residue 664 (I664V, I664Y) and at residue 690 (Y690F, Y690I, Y690V). In addition, we will incorporate a novel screening assay to identify key residues within Ad pol that will alter dNTP utilization. To do this, we will use the Pfu DNA polymerase as a model system to effectively screen dNTP binding mutations. *To be jointly performed by Drs. Kim and Dewhurst.*
B. Expression of mutated polymerase proteins in insect cells using a baculovirus vector system, and purification of the polymerase from those cells. (Months 3-12). *To be jointly performed by Drs. Dewhurst and Kim . Dr. Dewhurst will make and propagate the baculovirus vectors; Dr. Kim will biochemically purify the recombinant adenovirus polymerase proteins.*
C. Biochemical characterization of the catalytic activity and dNTP binding affinity of the purified polymerase enzymes over a range of different substrate (dNTP) concentrations. (Months 4-12). *To be performed by Dr. Kim.*

Task 2. To generate replication-competent adenovirus type-5 (Ad5) vectors containing mutated DNA polymerases with reduced dNTP binding affinity, and to assess vector replication in vitro. (Months 6-18).

A. Mutagenesis of the polymerase gene within full-length, replication-competent Ad5 vector backbones. (Months 6-13). *To be performed by Dr. Dewhurst.*

B. Generation, purification and titration of Ad5 vector stocks (Months 8-15). *To be performed by Dr. Dewhurst.*
C. Quantitative analysis of vector replication and host cell cytolysis in a panel of breast cancer cells lines and in primary human mammary epithelial cells. (Months 10-18). *To be jointly performed by Drs. Dewhurst and Kim; Dr. Dewhurst will measure host cell killing while Dr. Kim will measure cellular dNTP content and perform qPCR analysis for viral DNA load.*

Task 3. To evaluate the effectiveness of replication-competent adenovirus type-5 (Ad5) vectors containing mutated DNA polymerases with reduced dNTP binding affinity in vivo. (Months 13-24). To be initiated in Year Two.

Research Accomplishments associated with the above tasks

Task 1. To identify mutated derivatives of the adenovirus DNA polymerase (Ad pol) that have a substantially reduced dNTP binding affinity, but normal catalytic activity under conditions of substrate excess.

a. Mutation of selected polymerase residues within Ad pol using site-directed mutagenesis.

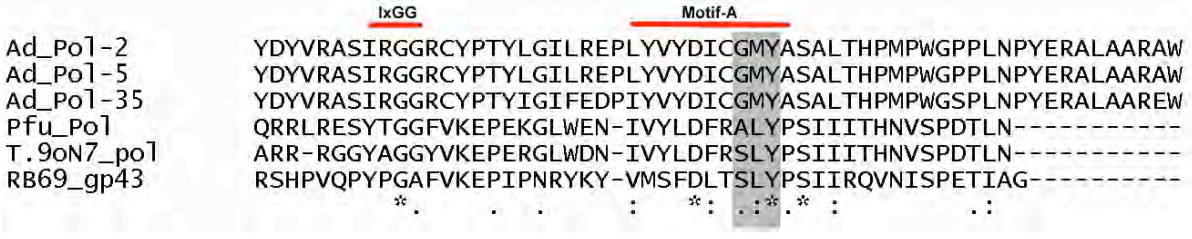
Construction of Ad5 replication competent viral molecular clones

We have created a series of conservative substitutions at pol residues known to affect dNTP utilization. Mutations include conservative changes at residue 664 (I664V, I664Y) and at residue 690 (Y690F, Y690I, Y690V). All of these mutants have been inserted into the backbone of a replication competent (E1+) Ad5 vector (Task 2).

Use of a novel screening assay to identify novel Ad5 pol residues that affect dNTP binding and utliization: Pfu as a model for Ad Pol

We are using a novel screening assay to identify key residues within Ad pol that will alter dNTP binding and utlization. This approach relies on the high degree of conservation of the core dNTP binding motifs between Ad pol and Pfu pol (F**Igure 1**).

FIgure 1: Ad pol and Pfu pol share core dNTP binding motifs

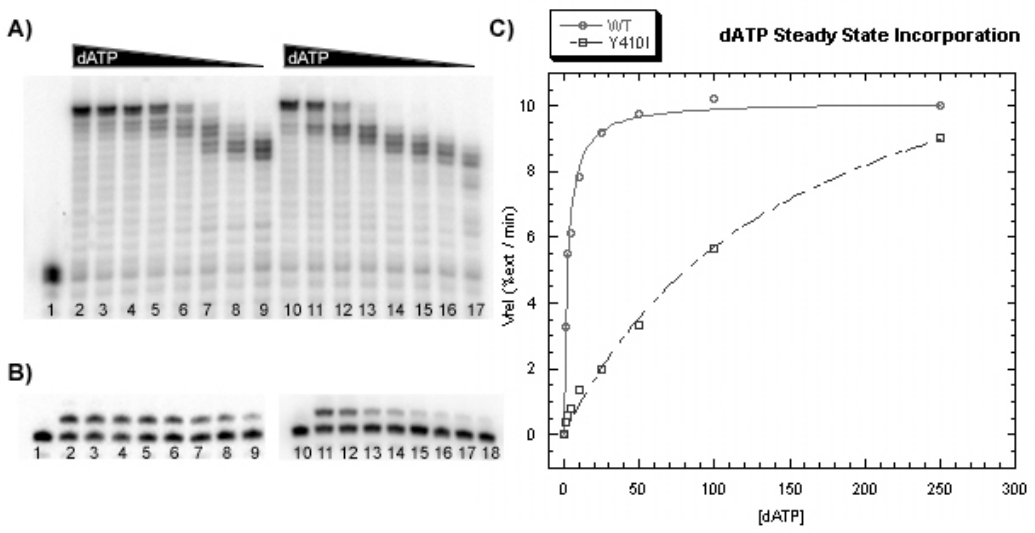


LEGEND: *Clustal-W alignment of adenovirus DNA polymerase core motifs, along with sequences from selected thermostable DNA polymerases (including Pfu DNA polymerase) and from the RB69 bacteriophage. The area of interest to this study is highlighted in grey.*

The conserved Tyrosine in Motif A is known to interact with the incoming dNTP in many different polymerases (including Vent pol, phi29 bacteriophage polymerase and MuLV RT). We therefore theorized that alterations of this region of Pfu Pol might modulate dNTP binding affinity, and we constructed a mutant version of the Pfu DNA polymerase in which the conserved Y410 residue and its neighbors were non-conservatively substituted.

The Y410I Pfu polymerase was found to have a profound defect in dNTP utilization at lower concentrations in both multi-nucleotide and single nucleotide extension assays, when compared to the wild-type (WT) protein (**Figure 2**). To better analyze our single nucleotide extension we quantified and fit it to the Michaelis-Menton equation with nonlinear regression which was later confirmed by Lineweaver Burke double reciprocal plots for representative experiments (**Table 1**).

Figure 2: A Y410I mutant of Pfu DNA polymerase is defective in its ability to use dNTPs



LEGEND: Steady state reactions were carried out at 55° C for 5 minutes with varying amounts of all four dNTP's or dATP. Negative controls (no enzyme; lane 1) indicate the position of the unextended primer. Concentrations of dNTP/dATP for each of the eight reactions were as follows: 250,100,50,25,10,5,2.5,1 µM. **A)** Representative multi-nucleotide dNTP titration for WT Pfu Pol 3'-5' exo - (lanes 2-9) and Pfu Pol Mutant Y410I (lanes 10-17) **B)** Representative single nucleotide extension (dATP) titration for WT Pfu Pol (lanes 2-9) and Y410I (lanes 11-18) **C)** Nonlinear regression fit of of the Michaelis Menton equation to the single nucleotide extension preformed in duplicate for WT (circles) and Y410I (squares).

In addition to the L408I mutant, we also constructed and tested a series of other single amino acid substitution mutants of the Pfu DNA polymerase. The results are summarized in **Table 1**.

As shown in **Table 1**, we observed a 20-61 fold difference in K_m when comparing the wild-type Pfu DNA polymerase to our panel of Y410 mutants. Substitution of the neighboring L409 residue had a more subtle effect relative to WT, resulting in a roughly 5-fold difference in K_m. Several substitutions of the adjacent A408 residue (A408 -> G,C or V) resulted in complete loss of enzymatic activity; the only functional active A408 substitution mutant was the conservative A408S mutant, which had a 4-fold difference in K_m.

We constructed a series of mutations at the corresponding residue in the adenovirus polymerase, and then tested their ability to confer a conditionally-replicative phenotype on an adenovirus vector (see Task 2). Mutants constructed included: (1) mutants of the Y690 residue in Ad pol (equivalent to Pfu pol Y410; Ad pol mutants constructed were Y690A, Y690F and Y690I); (2) mutants of the adjacent residues in Ad pol (equivalent to Pfu pol L409 and A408; Ad pol mutants constructed were M689V, M689I and G688S).

b. Expression of mutated polymerase proteins in insect cells using a baculovirus vector system, and purification of the polymerase from those cells.

Progress:

- Ad5 Exonuclease-deficient (Exo-) DNA Polymerase (wild-type): The DNA sequence encoding the full length Exo+ Ad5 DNA polymerase has been cloned into the baculovirus pAcgp67A vector, and subjected to site-directed mutagenesis to eliminate its intrinic exonuclease activity (key exonuclease active site residues have been substituted with alanines; a total of 4 point mutations were introduced to do this: D283A and E285A (domain I) plus Y580A and D584A (domain III). Efforts to generate baculovirus stocks expressing this modified Ad

Table 1 : Pfu Pol Mutant Steady State Parameters

Pfu Pol mutant (exo-)	**Km (μM)**	**kcat (s-1)**	**kcat / Km** (μM-1 s-1)	**~ [Pfu Pol]** (nM)
WT	2.48	0.2736	0.11035	0.122
Y410L	68.62 **(x 27)**	0.0042	0.00006 **(x 1780)**	11.000
Y410I	152.43 **(x 61)**	0.0016	0.00001**(x 10364)**	29.333
Y410V	50.48 **(x 20**)	0.0638	0.00126 **(x 87)**	0.550
L409I	12.77 **(x 5)**	0.0485	0.00381 **(x 29)**	0.688
L409V	12.06 **(x 5)**	0.0296	0.02969 **(x 45)**	1.048
A408S	9.91 **(x 4)**	0.2742	0.27424 **(x 4)**	0.122

pol protein are underway.

- Ad5 Exo- DNA Polymerase (I664V mutant): A DNA construct encoding this mutated DNA polymerase is under construction, and will be expressed in the baculovirus system.

- Ad5 pTP and DBP (wild-type): DNA sequences encoding the Ad5 terminal protein (pTP) and DNA-binding protein (DBP) have been cloned into pAcgp67A baculovirus vector. Efforts to generate baculovirus stocks expressing this modified Ad pol protein are underway.

c. *Biochemical characterization of the catalytic activity and dNTP binding affinity of the purified polymerase enzymes over a range of different substrate (dNTP) concentrations.*

Progress: Not yet initiated. Emphasis has been placed on necessary preliminary tasks (A & B above)

Task 2. To generate replication-competent adenovirus type-5 (Ad5) vectors containing mutated DNA polymerases with reduced dNTP binding affinity, and to assess vector replication in vitro.

a. *Mutagenesis of the polymerase gene within full-length, replication-competent Ad5 vector backbones.*

Progress: We have created a series of conservative substitutions at pol residues known to affect dNTP utilization: I664V, I664Y as well as Y690F, Y690I, Y690V. All of these mutants have been introduced into the backbone of a replication competent (E1+) Ad5 vector. Of the viruses created in this manner, none were viable - with the exception of the I664V mutant, which was found to be replication-competent. Efforts to characterize the I664V mutant virus are ongoing.

Table 1: Polymerase mutants and status of virus construction

Mutation	Viral Polymerase Motif	Description of Mutation	Progress
I664S	IxGG Motif	Expected to disrupt interaction of template DNA and polymerase	Non-viable virus mutant
I664Y	IxGG Motif	“”	Non-viable virus mutant
I664V	IxGG Motif	“”	Replication-competent viral molecular clone
I664M	IxGG Motif	“”	Virus under construction
I664F	IxGG Motif	“”	Virus under construction
Y690A	Motif A	Expected to disrupt polymerase-dNTP interaction	Non-viable virus mutant
Y690F	Motif A	“”	Non-viable virus mutant
Y690I	Motif A	“”	Non-viable virus mutant
Y690V	Motif A	“”	Non-viable virus mutant
G688S	Motif A	Identified by screening of Pfu DNA polymerase; expected to disrupt polymerase-dNTP interaction	Virus under construction
M689I	Motif A	“”	Virus under construction
M689V	Motif A	“”	Virus under construction

b. Generation, purification and titration of Ad5 vector stocks.

Progress: Not yet initiated. Emphasis has been placed on necessary preliminary tasks (A above)

c. Quantitative analysis of vector replication and host cell cytolysis in a panel of breast cancer cells lines and in primary human mammary epithelial cells. (Months 10-18).

Progress: Not yet initiated. Emphasis has been placed on necessary preliminary tasks (A above)

Task 3. To evaluate the effectiveness of replication-competent adenovirus type-5 (Ad5) vectors containing mutated DNA polymerases with reduced dNTP binding affinity in vivo.

Progress: This Task was scheduled for year two.

Modified Statement of Work

In light of the need to extend the unanticipated complexity of Task 1 (noted above), we therefore propose a **modified Statement of Work**, as outlined below (note that the major change here is a revision of the timeline, not the actual work).

1. **Task One (scope unchanged):** To identify mutated derivatives of the adenovirus DNA polymerase (Ad pol) that have a substantially reduced dNTP binding affinity, but normal catalytic activity under conditions of substrate excess. *Original Timeline: months 1-12; Modified Timeline: Will extend in year two (at least to month 18)*.

2. **Task Two (scope unchanged):** To generate replication-competent adenovirus type-5 (Ad5) vectors containing mutated DNA polymerases with reduced dNTP binding affinity, and to assess vector replication in vitro. *Original Timeline: months 6-18; Modified Timeline: Will extend at least to month 24.*

3. **Task Three (unchanged):** To evaluate the effectiveness of replication-competent adenovirus type-5 (Ad5) vectors containing mutated DNA polymerases with reduced dNTP binding affinity in vivo. *Original Timeline: months 13-24; Modified Timeline: Will be initiated during a projected no-cost extension period (months 25-36).*

IMPORTANT NOTE: Because of the fact that it will be essential to derive adenovirus vectors that selectively replicate in low dNTP environments (such as tumor cells) before we can proceed with in vivo experiments, this Task (Tasks 1, 2) will remain our major focus. This is a scientific necessity. Should progress prove slower than anticipated, it is conceivable that we may not initiate the in vivo studies (Task Three). Should that prove to be the case, we will consider a future grant application to undertake this work.

KEY RESEARCH ACCOMPLISHMENTS

Current Project Year

- We have used site-directed mutagenesis to create a panel of modified adenovirus polymerase genes in which key amino acid residues known to affect dNTP utilization have been replaced by other amino acids (using both conservative and non-conservative substitutions).

- We have developed a new screening assay to identify adenovirus polymerase protein residues that may affect dNTP binding and utilization. This approach uses the thermostable DNA polymerase, Pfu pol, as a model for the adenovirus polymerase. Pfu pol is ideal for this purpose because of the high degree of conservation of key polymerase residues, and also because it is a single subunit enzyme (and thus highly amenable to direct biochemical analysis, unlike the tripartite adenovirus DNA polymerase).

- Using the Pfu pol model, we have shown that amino acids that flank the highly conserved tyrosine within Motif-A of the core polymerase play an important role in dNTP binding and utilization by the enzyme (residues Y410, L409 and A408 in Pfu pol, which correspond to residues Y690, M689 and G688 in adenovirus polymerase).

- We have subcloned the genes encoding the adenovirus DNA polymerase (pol), terminal protein (pTP) and DNA-binding protein (DBP) into a baculovirus vector and initiated efforts to produce these three proteins in recombinant form in insect cells. We have also constructed an exonuclease-deficient mutant of the adenovirus DNA polymerase; this is expected to facilitate downstream biochemical characterization of the enzyme complex.

- We have introduced a series of mutated adenovirus polymerase genes into the backbone of a full-length, replication-competent Ad5 vector. We have then tested the ability of the resulting viruses to replicate in cultured mammalian cells. To date, seven mutants have been evaluated in this manner. Only one of these recombinant virus mutants has been found to be replication-competent (a I664V mutant); this virus is being actively characterized to determine possible selective replication in tumor cells. Several additional virus mutants are under construction and will be evaluated shortly.

REPORTABLE OUTCOMES

Manuscripts, abstracts, presentations: See Bibliography.

Patents and licenses applied for and/or issued: None

Degrees obtained that are supported by this award: None

Development of cell lines, tissue or serum repositories: None

Informatics such as databases and animal models, etc: None

Funding applied for based on work supported by this award: None

Employment or research opportunities applied for and/or received on experiences/training supported by this award:

Research training was provided for the following persons during the present project year:

Ms. Cristina Capella-Gonzalez, a Ph.D. trainee in the Dewhurst laboratory. Ms. Capella is presently conducting her thesis research at the University of Rochester Medical Center, under the supervision of Dr. Dewhurst. She is a member of an under-represented minority group.

Additional personnel will be added to the project in year two; we experienced hiring delays in year one.

CONCLUSIONS

The conclusions which can be drawn from the first year of our experiments are as follows:

1. Pfu DNA polymerase can be used as a valid surrogate for the adenovirus DNA polymerase, in order to facilitate the identification of amino acid residues that affect dNTP binding and utilization.

2. Amino acids that flank the highly conserved tyrosine within Motif-A of the core polymerase play an important role in dNTP binding and utilization by the enzyme (residues Y410, L409 and A408 in Pfu pol, which correspond to residues Y690, M689 and G688 in adenovirus polymerase).

3. Mutations that drastically alter the dNTP binding efficiency of the adenovirus DNA polymerase give rise to replication-defective viruses, when substituted into the backbone of an intact adenovirus vector. In contrast, some mutations with more modest effects on dNTP binding efficiency (e.g., the I664V mutation) are compatible with virus replication and permit the recovery of infectious virus.

"So What Section"

Our data advance the goals of this grant application, and bring us closer to being able to test our underlying hypothesis, that adenovirus vectors with mutated DNA polymerase genes may represent a powerful new approach for permitting selective vector replication in breast cancer cells, thereby killing breast cancer cells while sparing normal tissue.

BIBLIOGRAPHY (PUBLICATIONS)

These materials are all also included as Appendices

Abstract:

C. Capella, B. Kim, S. Dewhurst**.** New conditionally replicating adenovirus vectors for breast cancer therapy. Era of Hope meeting, June 2008 (Baltimore, MD).

- 2 pages

APPENDIX MATERIALS

Award Number:	W81XWH-071-0376
TITLE:	New Conditionally Replicating Adenovirus Vectors for Breast Cancer Therapy
PRINCIPAL INVESTIGATOR:	Stephen Dewhurst, Ph.D.
CONTRACTING ORGANIZATION:	University of Rochester ROCHESTER, NEW YORK 14627
REPORT DATE:	September, 2008
TYPE OF REPORT:	Annual

List of Materials Appended

New Manuscripts:

None

Abstract:

C. Capella, B. Kim, S. Dewhurst**.** New conditionally replicating adenovirus vectors for breast cancer therapy. Era of Hope meeting, June 2008 (Baltimore, MD).

- 2 pages

ABSTRACT

C. Capella, B. Kim, S. Dewhurst**.**

New conditionally replicating adenovirus vectors for breast cancer therapy.
Era of Hope meeting, June 2008 (Baltimore, MD).

Abstract

Background and Objectives: Breast cancer is the most common form of cancer in the United States, and the second leading cause of cancer deaths among women. As a result, there is a pressing need to develop new treatments. Oncolytic, conditionally-replicating adenoviruses (CRADs) represent one such approach. While initial clinical studies of oncolytic CRADs have shown that these vectors are generally safe and well tolerated, additional improvements are needed. Our objective to develop new CRADs containing mutant DNA polymerases with a high functional dNTP requirement; we hypothesize that these vectors will replicate selectively in tumor cells.

Brief Methodologies: Standard mutagenesis techniques were used to construct Adenovirus type 5 vectors with selected mutations in the viral polymerase gene. The replication characteristics of these vectors were then assessed in cultured cells, using expression of a vector-encoded reporter gene (GFP) as a surrogate for virus replication.

Results to Date: Mutations were made to key adenoviral polymerase motifs that are expected to interact with the dNTP substrate. Replication-competent virus molecular clones that contain these engineered mutations were constructed, and their replicative properties were evaluated in tumor-derived cell lines and in cells that were supplemented with exogenous deoxynucleosides (as a way to elevate intracellular dNTP levels). Out of an initial panel of 7 polymerase mutant virus constructs, one was

found to be replication-competent (I664V). Transgene (GFP) expression from this vector was enhanced when cells were supplemented with exogenous deoxynucleosides. This suggests that the presence of additional dNTPs within the cellular environment either enhances viral replication or increases transgene expression at a transcriptional level.

Conclusions: Non-conservative mutagenesis of several selected polymerase motifs appears to compromise virus replication, while a conservative mutation within a conserved IxGG motif (I664V) was tolerated and gave rise to replication-competent virus. We are presently testing additional mutants, with a view to identifying viruses that replicate selectively in high dNTP environments, such as those found in breast cancer cells. It is expected that these new adenovirus vectors will provide an important tool for breast cancer treatment.